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SIMULATING THE ACTIVITY OF VINCRISTIN (AN ANTICANCEROUS DRUG) WITH BIOGENIC NANOPARTICLES USING *VIGNA RADIATASEEDS*

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ABSTRACT

Background: The present study reports the inhibitory effects of biogenic nanoparticles synthesized from *Tridax procumbens* stem (fresh and powder) aqueous extract on seed germination and seedling growth of *Vigna radiata*. These synthesized nanoparticles were well characterized by UV-visible spectroscopy; SEM; XRD and FTIR analysis. The constituents in the extract were also well characterized using FTIR analysis. The cytotoxic propertiesof the biogenic nanoparticles were then compared with the individual aqueous extract and vincristin (an anti-cancerous drug- used for ease in cancer.). The inhibition of seed germination and seedling growth was found to be dose-dependent and was suitable to quantify the bioactivity of biogenic nanoparticles preparations.

Objective: The present investigation, emphasizes on simulating the activity of the synthesized biogenic nanoparticles from *T. procumbens* withan anticancerous drug –vincristine using *in vitro* bioassay system (Kumar and Singhal, 2009). The growth promotion and/or retardation activity of crude plant extractis also described.

Method: After reviewing number of peer-reviewed published articles the safest and eco-friendly method for the synthesis of the nanoparticles had been adopted. Biogenic nanoparticles were later well characterized by SEM, XRD, and FTIR analysis. Their activity against anticancerous drug – vincristine, was tested using an *in vitro* bioassay on *Vigna radiata*.

Results: Present study brings out the role of biogenic nanoparticles in inhibiting seed germination which was dose dependent. With vincristin comparable inhibition was observed. The plant extract, bionanoparticles and vincristin, promoted water imbibitions by the seeds though their higher concentrations inhibited this process. Reduction in water imbibitions, caused by these failed to trigger processes leading to reduced seed germination and radicle decay. The growth retardation following these treatments could be attributed to the inhibition of cell division and radicle protrusion brought about by osmotic stress (de Castro et al., 2000). From these observations it could be safely inferred that the plant extracts exhibited effective inhibitory activity, though biogenic nanoparticles and vincristin were most effective at 1.0 and 2.0 mg ml^{-1}.

Conclusion: Biogenic nanoparticles are eco-friendly, cost effective, and rapidly synthesized, and hence are more acceptable than the anti-cancerous drugs available in the market. The latter have side effects, cost issues, toxic chemicals involved in their synthesis, storage complexities, etc. The present study opens new avenue for medical sciences where plant extract synthesized nanoparticles via “green route” can be the safer alternative than synthetic anti-cancerous products.

Key Words: Seed germination, Bioactivity, Seedling growth, Tridax procumbens, Vigna radiata, Vincristin, UV-visible, SEM, XRD, FTIR

INTRODUCTION

Nanotechnology chiefly concerns with the synthesis of nanoparticles of variable sizes, morphology, shapes and chemical composition and because of their unique physiochemical characteristics e.g. optical and electronic properties, catalytical activity, magnetic properties and antibacterial traits, they are being extensively researched for their potential applications in the field of life sciences.

The physical and chemical processes are generally employed for the manufacture of nanoparticles, but these methods are

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not ecofriendly and involve toxic metals in the synthesis process. These technical hitches in the nanoparticle synthesis are surmounted by biogenic process involving micro-mediated and plant-mediated route. Such a process is preferred because of simplicity, eco friendliness and biocompatibility. In the plant-mediated biological synthesis of nanoparticles, different plant constituents play a critical role in the reduction of Ag^+ to *\ silver nanoparticles.

Tridax procumbens Lin (Fam: Asteraceae) is a medicinally important plant found throughout India especially in Rajasthanwith several valuable chemical constituents e.g. flavone glycosides, chromone glycosides, sterols and polysaccharides with a Beta-1,6-D-galactan main chain. Further the ethyl acetate soluble fraction of hexane extract revealed trisbisbitiophene along with four terpenoids taraxasteryl acetate, lupeol, oleanolic acid and some flavonoids, etc. Unsaponiable fraction of petroleum ether fraction yielded campesterol, stigmasterol, beta-sitosterol. A number of pharmacological activities have been described by various investigators and these are furtherance of clotting time, e.g. wound healing, hepatoprotective, antimicrobial, anti-juvenile hormone, anti-inflammatory, anti-diarrhoeal, anti-protozoal, immunomodulatory, hair growth promoting, insecticidal, antioxidant activities, cardiovascular effects, etc. Additionally, its chemical constituents possess antimicrobial and immunomodulatory action (Malik et al., 2012).

Several chemicals, drugs and plant extracts are shown to have cytotoxic properties and are either used and /or proposed to be used for treating various cancers. These drugs have diverse modes of activity e.g. interference with cell-cycle kinetics, inhibition of proliferation of mitotically active cells by damaging the DNA during S-phase of the cell cycle or blocking the formation of mitotic spindle during the M phase or arrest mitotic activity.

Several natural products, having variable chemical structures, have been isolated as anti-cancerous agents. Srivastava et al. (2005) have reviewed plant-based anti-cancerous molecules and have discussed the chemical and biological profile of some significant lead molecules.These leads have variable effects ranging from interference with cell cycle kinetics, inhibition of the proliferation of mitotically active cells in variable ways e.g. damaging the DNA during S-phase of the cell cycle or blocking the formation of mitotic spindle during the M phase. Some plant derived lead molecules affect microtubules dynamics and signalling pathway causing mitotic arrest.

Many of the drugs exhibit anti-mitotic activity both *in vivo* and *in vitro* and are anti-cancerous. Currently, several anti-cancerous agents are available and there is a continuous search for new leads that may be more effective and harmless (Murthy et al., 2011).

Multiple bioassay systems and experimental models are used to decipher the anti-mitotic activity. These include bacterial cultures, *in vivo* investigations based on mice, root tip meristem etc. Most of these bioassays are cumbersome and time consuming. In recent years a simple *in vitro* bioassay has been successfully evaluated for the rapid and preliminary screening of anti-cancerous drugs (Kumar and Singhal, 2009).

In the present investigation, the synthesis of biogenic nanoparticles using *T.procumbens* has been undertaken and the growth promotion and/or retardation activity of crude plant extract, using *in vitro* bioassay system (Kumar and Singhal, 2009) is described. The activity is also compared with the action of the biogenic nanoparticles synthesized from the same source as well as anti-cancerous drug vincristin.

MATERIAL AND METHODS

Our current investigation was carried out at Jaipur National University and Indian Institute of Technology (IIT)-Kanpur.

Plant Material

Fresh branches of *T. procumbens* were collected from the campus of JNU, Jaipur.

Synthesis and characterization of Biogenic Nanoparticles

A known amount of stem(fresh and powder) of *T. procumbens*was added to 100 ml of triple deionized water individually and boiled for 15 min. The mixture was then filtered using WhatmanNo.1 filter paper to obtain aqueous extract of definite concentrations. For synthesis of nanoparticles, a known concentration of stem filtrate was interacted with different concentrations (1, 10, 15 and 20 mM) of $AgNO_3$ solution in a defined ratio (9:1) to make up 100 ml volume. The solution was incubated at room temperature for 1- 5 h and observed for the change in colour from greenish to brownish. This was followed by UV –vis. analysis (recording absorbance at 300 – 600 nm)using Genesys 10uv spectrophotometer. Further SEM, XRD and FTIR analyses were done (Kushwaha and Malik, 2012).FT-IR analysis of the sample was done using BRUKER-VERTEX-70 Model at a resolution of 4 cm^{-1} in KBr pellets. This analysis helped to reveal the capping agents responsible for biogenic synthesis.

Biogenic Nanoparticles - Stock Preparations

These synthesized biogenic nanoparticles werefurther used for making stocks comprising - 0.3, 0.5, 1.0 and 2.0mg ml^{-1}, respectively. Different dilutions (0.3, 0.5, 0.6, 0.8 and 1.0μl of stock/μl of distilled water) were prepared and used for evaluating the inhibitory effect of the biogenic nanoparticles on seed germination of *Vigna radiata*.

Plant Extract- Stock Preparations

Different quantities of fresh material (stem) and their powder(100, 130, 200, 400mg ml^{-1})were soaked and heated separately in on hot plate with distilled water for 15 min.The extracts were filtered separately with aWhatman No.1 filter paper to remove the suspended particles and the filtrate (the extract) was either used directly in the experiments or stored at 4°C until further use. Fivedilutions of individual stock extract were prepared (0.3, 0.5, 0.6, 0.8 and 1.0μl of stock/μl of distilled water) to make up the final volume of 300 μl using deionised water and were further used for testing cytotoxicity.

Seed germination

An inexpensive, simple quantitative assay was used for screening herbal aqueous extract for cyto-toxicity test as proposed by Kumar and Singhal (2009)and Murthy et al., (2011).

Seeds of *Vigna radiata* were obtained from Rajasthan State Seed Corporation, Durgapura, Jaipur. Uniformly selected seeds were sterilized with 5% NaOCl for 2 min and repeatedly washed under running tap water followed by distilled water.

15 seeds were sown in each Petri dish and incubated in BOD incubator set at 25° C. Petri dishes were irrigated with various test solutions. The data were sampled after 48h.Each experiment was run in triplicate and values represent their mean.

Drugvincristin (anticancerous) solutions were prepared as dilutions mentioned above and added to the filter paper in the Petri dishes. Petri dishes areirrigated with water(control) andanother onewith nanoparticlessolution and vincristin.

Care was taken to moisten the filter paper with control, drug and biogenic nanoparticlessolutions every 6 h. The length of the radicle (cm) was measured at the end of 48 h and percent mean values of the germinating seeds as well as seedling growth were evaluated inwater control and treated samples (extracts, biogenic nanoparticles and vincristin).

RESULTS AND DISCUSSION

Formation of Biogenic nanoparticles

Due to splitting of $AgNO_3$ into Ag^+ and NO_3^- change in colour of the reaction mixture was observed, with progressive time. Apparently the metabolites in the stem (fresh and powder) extract acted as e^- donor and reduce Ag^+ ions into Ag. Consequently, the formation of nanoparticles was indicated by brown colour of the aqueous solution following the excitation of surface plasmon vibrations **(Figure 1).**

Our findings substantiate the data from *Capsicum annum* (Li et al., 2007), *Aloe vera* extracts (Chandran et al., 2006), *Citrullus colocynthesis*(Satyavani et al., 2011)and *Boswellia ovalifoliolata*(Savithramma et al., 2011)except that in our instance we accomplished formation of nanoparticles at 5 and 10 mM of the aqueous solution.

Characterization of Biogenic nanoparticles

The reduction of silver ions during the incubation period is generally deciphered through UV-visible spectroscopy and this period is variable ranging from few minutes to several hours. During UV-visible analysis most of the absorbance peaks obtained with different sample used were located within a range of 420 nm which coincided with the results obtained with the extract of mangrove plant (leaf bud) with an absorbance peak at 426 nm (Umashankari et al., 2012). These peaks were obtained as in metal nanoparticles, conduction band and valence bands lie very close to each other and through these electrons are capable of making free movement. These free electrons give rise to Surface Plasmon Resonance (SPR) absorption band. SPR results due to the collective oscillations of electrons of synthesized nanoparticles in resonance with light waves **(Figure 2 (a) and (b)).**

Once the nanoparticles are synthesized they tend to agglomerate and the latter largely depends on the chemistry as well as the electromagnetic property. This agglomeration also depends on surface energy and thermodynamic instability of the synthesized Ag nanoparticles (Olenin et al., 2008). To prevent their agglomeration the synthesized nanoparticles can be coatd with non-magnetic substances or different types of stabilizing agents can be used including PEG (used in the present studies) . The synthesized biogenic nanoparticles obtained were in an aqueos from which was converted into powder through the process of lyophilization **(Figure 3 (a) and (b)).**

Further characterization of synthesized biogenic nanoparticles is ascertained using SEM and XRD (Kushwaha and Malik, 2012a; Kushwaha and Malik, 2013). Biogenic nanoparticles from the powder extract of *Tridax* plant organs (stem) is already reported where the synthesized nanoparticles are of size ranging from 35.44 nm (Kushwaha and Malik, 2012) while from the fresh stem extract nanoparticles of an average size 21.84 is obtained **(Figure 3 (c) and (d); Figure 4 (a) and (b) respectively).**

Figure 5 (a) and (b)shows the graph of FTIR analysis of stem extract and the data on corresponding wave number and resultant group are set in table 1.As is evident the resultant groups varied and of special mention are aldehydes, primary amines, amides, lactones, aliphatic amines, alkanes, etc. **Figure 5 (a) and (b)and table 1**

Cytotoxicity test

Seed germination and seedling growth assay was employed to decipher the cytotoxicity of synthesized nanoparticles**.**

Seeds germination was 100% in the water control under the test conditions. Aqueous extract (fresh) of stem caused a dose-dependent inhibitory effect on seed germination (table 2 and Figure 6). With 100mg ml^{-1} stem extract (fresh) stock solution, 100% seed germination was observed at 0.3 and 0.5µl of stock/µl of distilled waterdilutions while 0.6*µl* of stock/*µl* of distilled watercaused 75% germination. At higher concentrations (0.8 and 1.0*µ*l of stock / *µ*l of distilled water) of stock dilutions seed germination was completely inhibited. With stem extract (powder) 100% germination was observed at 0.3, 0.5 and 0.6 *µl* of stock/*µl* of distilled waterdilutions and seed germination was 75% at 0.8 and 1.0*µl* of stock/*µl* of distilled waterdilutions. At higher concentration of stocks (130 and 200 and 400mg ml^{-1})obtained from stem extracts seeds imbibed water but failed to germinate(table 2 and Figure 6)

Table 3 shows data on biogenic nanoparticles,synthesized from fresh and powder extract of the same with varying concentrations (0.3, 0.5, 1.0 and 2.0mg ml^{-1}). Both inhibited seed germination and seedling growth (Figures 6 and 7). Inhibition was evident even at low concentrations. Fresh extract of stem even atlow concentration wascapableof interferingwith seed germination.In brief, at low concentrations, nanoparticles effectively arrested seed germination and possibly exhibited anti-mitotic behaviour. **Figures 6 and 7, Table 2 and Table 3**

In another experiment, we compared the effect of biogenic nanoparticles,with an anti-cancerous drug (vincristin). The data revealed that the effect with vincristin was dose dependent but seed germination was totally absent at the stock concentrations used. When the data from biogenic nanoparticles were compared with vincristin it was found that the results obtained with 1 mg ml^{-1} of biogenic nanoparticles (powder stem extract) were more inhibitorythan the vincristin at the same stock concentration.

Interestingly, the effect of fresh stem extract (400 mg ml^{-1}) was nearly the same asvincristin (1.0mg ml^{-1}). Biogenic nanoparticles (fresh stem extract) at 2.0mg ml^{-1} of stock concentration were more effective than 1.0mg ml^{-1} of vincristin , whereas1.0mg ml^{-1} of the biogenic nanoparticles was nearly as effective as 1.0mg ml^{-1} of vincristin.

The efficacy of this assay system in screening inhibitory activity of stem extract along with nanoparticles in *T. procumbens* has been compared with an established anti-cancerous drug vincristin.

There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the progress of complications associated with diseases (Rose et al., 1982). The important aspects that have shifted the interest towards the naturally occurring antioxidants are the toxic and/or mutagenic effects of synthetic antioxidant components. Numerous phyto-constituents are shown to be concerned with free radical scavenging or antioxidant activity (Aruoma and Cuppett, 1997). Flavonoids and other phenolic compounds (hydroxyl cinnamic derivatives, catechines, etc.) have been reported as scavengers and inhibitors of lipid peroxidation (Formica and Regelson, 1995).

There are many interesting ways of isolating active principles from the drugs. In the present investigation successful identificationthrough*in vitro* assay was performed using the plant extract (fresh and powder). Many other methods and system are available through which inhibition of growth specified cancer cell lines in tissue culture have been studied (Tominagaa et al, 1999; Skehan et al., 1990). In, a recent report an inexpensive assay was performed using sprouting parameters in green gram (Kumar and Singhal, 2009), using plant extract of different stocks with variable dilutions were used.

In the present study, the phyto-constituents of the extract obtained from *T. procumbens* were identified using FTIR analysis, which revealed the chemical composition of the plant source. Further it aided, in identifying the compounds possibly responsible for the inhibitory behaviour. As, stated earlier the presence of alkanes, aldehydes, ketones, phenols, aliphatic amines and lactones was identified. It has been reported that the inhibitory effect of long chain alcohols, aldehydes, ketones increased with enhanced lypophilicity, as they amplified the solubility across the cell membrane. These constituents are most effective against seed germination and early seedlings growth. The lead compounds are reported to interfere with the metabolic processes concerned with energy regulating organelles (chloroplast and mitochondria) or with the cell division process (microtubule organization). Some, workers have reported that lactones inhibit germination of seeds at concentrations of 250-300 ppm (Orcutt and Nelson, 1996). The available information on chemical constituents tempts one to explain the inhibitory behaviour caused by the plant extract.

Presently we have employed germinating seeds bioassay to compare the response against plant extracts, biogenic nanopartices and vincristin. It may be added that seed germination comprises various phases e.g. water imbibitions following soaking of seeds in various chemicals as well as biogenic nanoparticles. The quiescent embryo begins to grow due to water imbibitions, leading to cracking of seed coat and protrusion of radicle. Clearly the imbibitions by seeds are followed by concomitant metabolic activity of embryo and active cell division leading to seedling growth.

As is evident, the reduction in seed germination was associated with increase in extract concentrations as well as biogenic nanoparticles. At highest concentrations of the two (plant extracts and biogenic nanoparticles) the retardation of germination could be compared with the one caused by vincristin. From this it is inferred that the drug as well as biogenic nanoparticles affected water uptake and hence osmotic potential of the seeds causing reduction in turgid pressure within the seed and precluded the radicleprotrusion.

We observed that fresh extract of stem was comparatively moreinhibitory arrestingvarious stages of seed germination. When the dilution decreased from 0.3 to 1μl of stock/μl of distilled waterusing fresh stem stock, the retardation of seed germination was most pronounced, whereas powder extract was less effective.

This inhibiting capability of the stem extract of *T. procumbens*using seed germination bioassay is demonstratedfor the first time.

Present studyalso brings out the role of biogenic nanoparticles in inhibiting seed germination which was dose dependent. With vincristin comparable inhibition was observed.

The plant extract, bionanoparticls and vincristin, promoted water imbibitions by the seeds though their higher concentrations inhibited this process.Reduction in water imbibitions,caused by these failed to trigger processes leading to seed germination and radicle decay. The growth retardation following these treatments could be attributed to the inhibition of cell division and radicle protrusion brought about by osmotic stress (de Castro et al., 2000). From these observations it could be safely inferred that the plant extracts exhibited effective inhibitory activity, thoughbiogenic nanoparticles and vincristin were most effective at 1.0 and 2.0mg ml^{-1}.

CONCLUSION

Biogenic nanoparticles are more eco-friendly, cost effective, and rapidly synthesized, and hence are more acceptable than the anti-cancerous drugs available in the market. The latter have side effects, cost issues, toxic chemicals involved in their synthesis, storage complexities, etc. Change in colour of the reaction mixture during the time of incubation indicated the formation of silver nanoparticles and was confirmed by the characteristic peaks obtained by UV–visible spectra analysis. The size of particles was well characterized by SEM and XRD analysis. It can be predicted that the formation of nanoparticles occurs due to the presence of lactone, ketones, phenols and amines, which are abundantly found in *T. procumbens* and are also confirmed from the FTIR spectra.

The present study opens new avenue for medical sciences where plant extract synthesized nanoparticles via "green route" can be the safer alternative than synthetic anti-cancerous products.

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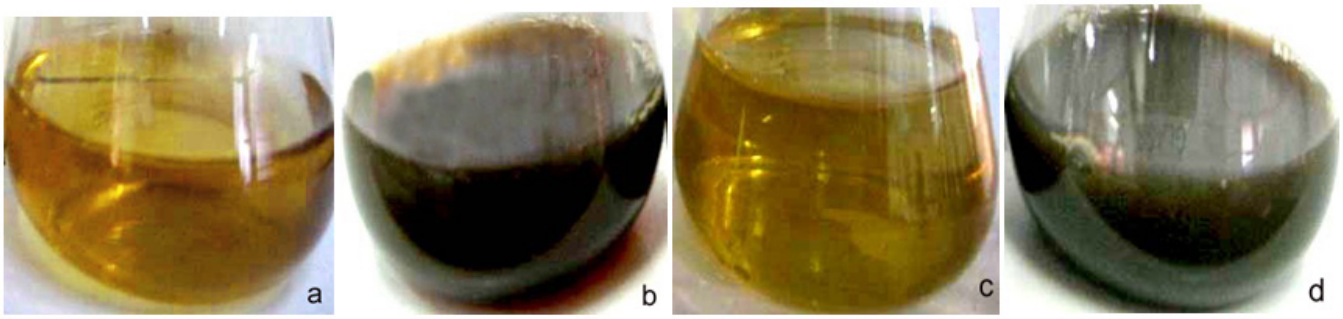

Figure 1 (a)-(d): *T. procumbens* (a) and (b) change in colour of the powdered stem reaction mixture (PSRM) (5 mM of $AgNO_3$).*T. procumbens* (c) and (d) change in colour of the fresh stem reaction mixture (FSRM) (10 mM of $AgNO_3$).

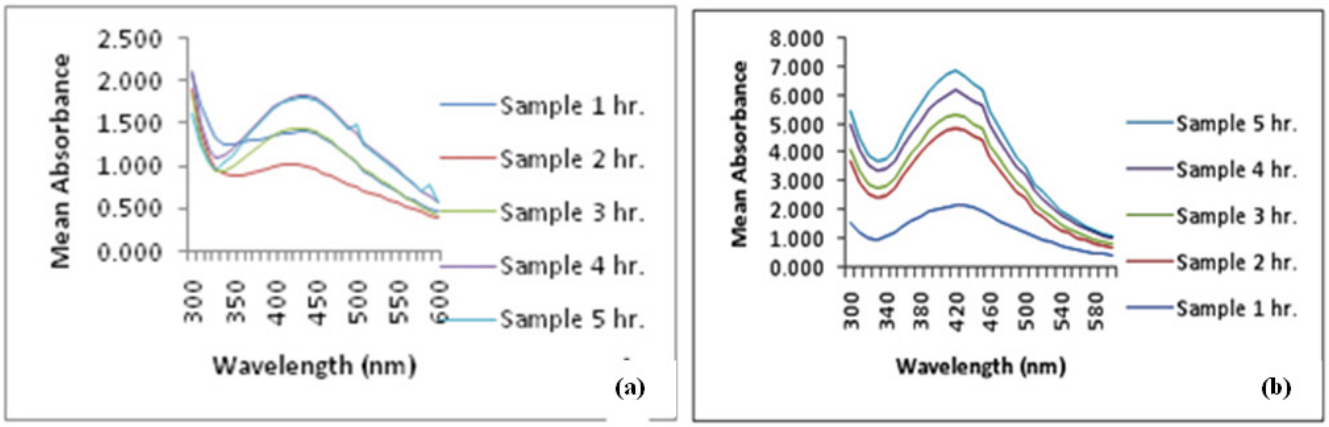

Figure 2 (a)-(b): Showing the peak values of the synthesized TNP using stem (powder and fresh material and $AgNO_3$ (5 and 10 mM respectively).

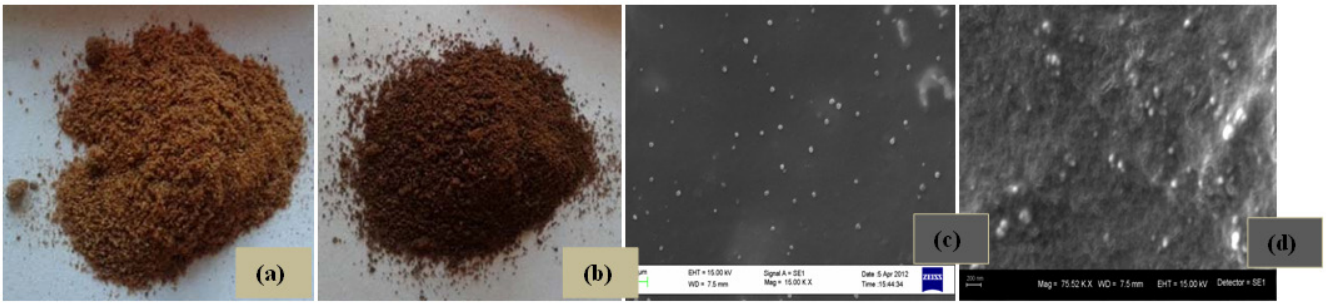

Figure 3 (a)–(d): (a) and (b) represents the powder of the synthesized biogenic nanoparticles through lyophilisation; (c) and (d) represents the SEM Images of the synthesized biogenic nanoparticles.

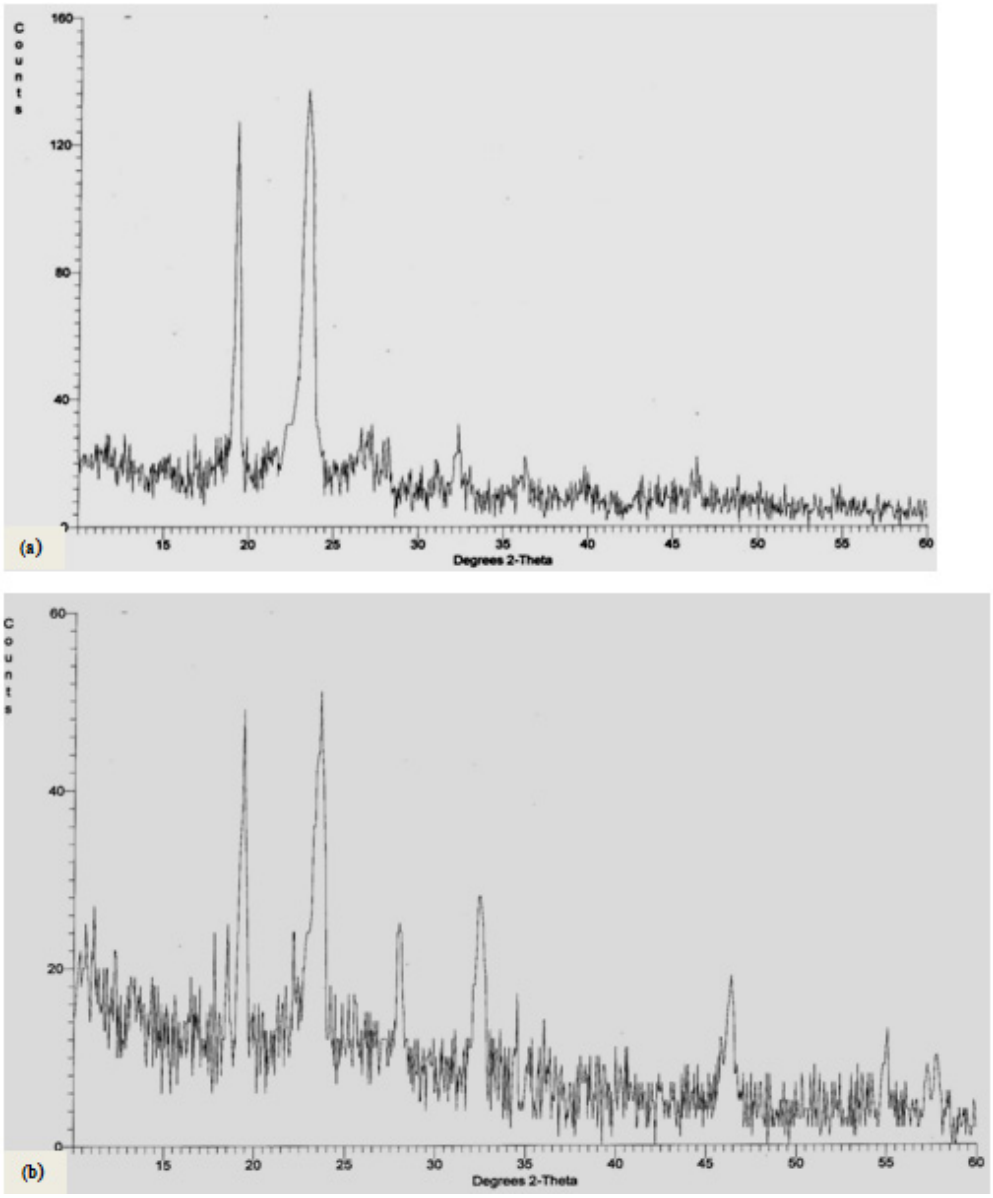

Figure 4 (a) and (b): Showing the results of XRD Analysis of the biogenic nanoparticles synthesized using powder and fresh material of *T. procumbens* respectively.

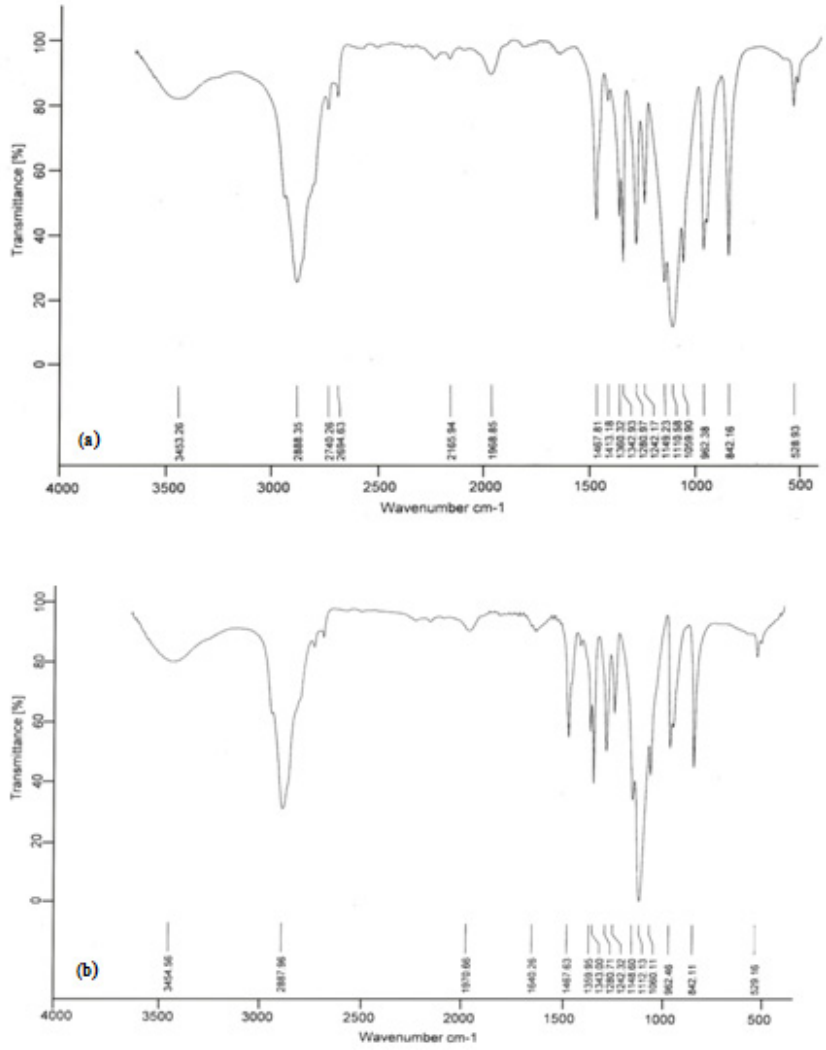

Figure 5: (a) and (b) representing the graphs of FTIR-Analysis of biogenic nanoparticles.

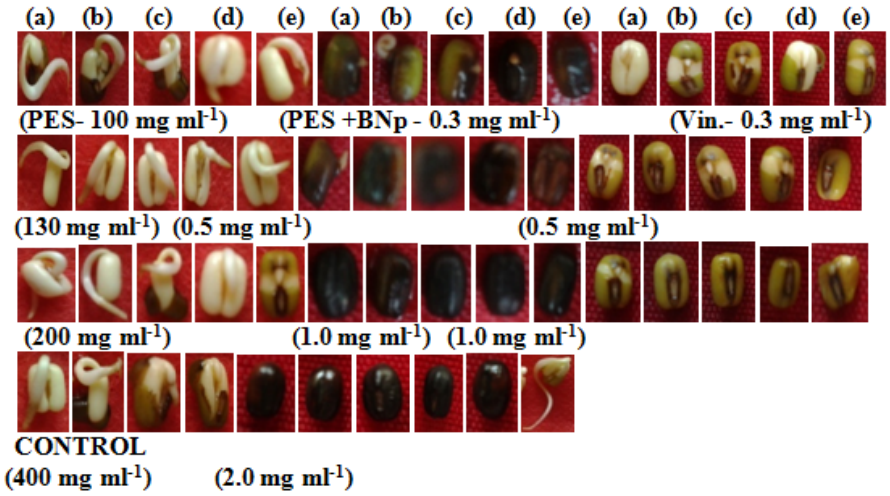

Figure 6: Seed germination, seedling emergence and seedling growth as affected by powder extract of stem (Left), biogenic nanoparticles (Middle) and vincristin (Right) using various dilutions - (a)-0.3 *µ*l/ µl, (b) 0.5 *µ*l/ *µ*l, (c) 0.6 *µ*l/ *µ*l, (d) 0.8 *µ*l/ *µ*l and (e) 1.0 *µ*l/ *µ*l and compared with control.

**µ*l/ µl stand for micro litres of stock solutions per micro litres of distilled water

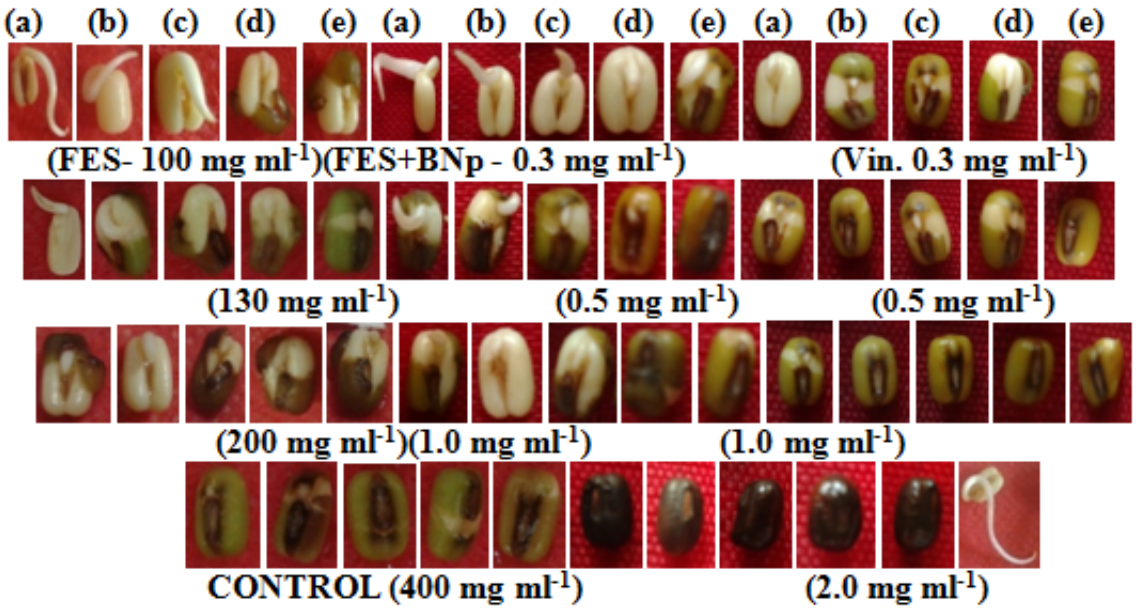

Figure 7: Seed germination, seedling emergence and seedling growth as affected by fresh extract of stem (Left), biogenic nanoparticles (Middle) and vincristin (Right) using various dilutions - (a)-0.3 *µl/ µl*, (b) 0.5 *µl/ µl*, (c) 0.6 *µl/ µl*, (d) 0.8 *µl/ µl* and (e) 1.0 *µl/ µl* and compared with control.
**µl/ µl* stand for micro litres of stock solutions per micro litres of distilled water.

Table 1: FTIR analysis of stem extracts–(fresh and powder)

Corresponding wave-number (cm^{-1})	Resultant group
3400-3200	-OH stretching of alcohols and phenols
2887.50	Aldehyde stretching of alkanes and primary amines
1640-1550	N-H bends of primary and secondary amides
1450-1375	C-H (C-H_3 bends) of alkanes
1350-1000	C-N stretching vibration of amines or C-O stretching of alcohols, ethers, carboxylic acid, esters and anhydrides
1110.58 and 1109.56	Lactones
842.16 and 842.21	Aliphatic amines, Alkanes

Table 2: Showing data of % seed germination of T. procumbens stem (fresh and powder) extract

Stocks (mg ml^{-1})	% seed germination using various dilutions (µl of stock/µl of deionized water dilutions)									
Stem	0.3		0.5		0.6		0.8		1	
	FS	PS	FS	PS	FS	PS	FS	PS	FS	PS
100	100	100	100	100	25	100	NIL	75	NIL	50
130	NIL	100	NIL	66.6	NIL	50	NIL	50	NIL	25
200	NIL	100	NIL	100	NIL	66.6	NIL	25	NIL	NIL
400	NIL	100	NIL	75	NIL	66.6	NIL	NIL	NIL	NIL

(FS- Fresh Stem; PS- Powder Stem)

Table 3: Showing data of % seed germination of T. procumbensstem (fresh and powder) extract derived BNPs and anti-cancerous drug –vincristin

Stocks (mg ml^{-1})	% seed germination using various dilutions (µl of stock/µl of deionized water dilutions)									
BNPs Stem	**0.3**		**0.5**		**0.6**		**0.8**		**1**	
	FS	PS	FS	PS	FS	PS	FS	PS	FS	PS
0.3	100	NIL	50	NIL	25	NIL	NIL	NIL	NIL	50
0.5	50	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	25
1	25	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL
2	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL
Vincristin 0.3, 0.5 and 1 (mg ml^{-1})	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL

(FS- Fresh Stem; PS- Powder Stem; BNP-Biogenic Nanoparticle)